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The *Hansenula polymorpha PER9* Gene Encodes a Peroxisomal Membrane Protein Essential for Peroxisome Assembly and Integrity*

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We have cloned and characterized the *Hansenula polymorpha PER9* gene by functional complementation of the *per9-1* mutant of *H. polymorpha*, which is defective in peroxisome biogenesis. The predicted product, Per9p, is a polypeptide of 52 kDa with sequence similarity to Pas3p, a protein involved in peroxisome biogenesis in *Saccharomyces cerevisiae*. In a *per9* disruption strain (Δ*per9*), peroxisomal matrix and membrane proteins are present at wild-type levels. The matrix proteins accumulated in the cytoplasm. However, the location of the membrane proteins remained obscure; fully induced Δ*per9* cells lacked residual peroxisomal vesicles ("ghosts"). Analysis of the activity of the *PER9* promoter revealed that *PER9* expression was low in cells grown on glucose, but was enhanced during growth of cells on peroxisome-inducing substrates. The highest expression levels were observed in cells grown on methanol. Localization studies revealed that Per9p is an integral membrane protein of the peroxisome. Targeting studies suggested that Per9p may be sorted to the peroxisome via the endoplasmic reticulum. Overexpression of *PER9* induced a significant increase in the number of peroxisomes per cell, a result that suggests that Per9p may be involved in peroxisome proliferation and/or membrane biosynthesis. When *PER9* expression was placed under the control of a strongly regulatable promoter and switched off, peroxisomes were observed to disintegrate over time in a manner that suggested that Per9p may be required for maintenance of the peroxisomal membrane.

Peroxisomes are cell organelles that are present in virtually all eukaryotic cells. They perform specific metabolic functions that are often related to the developmental stage and/or the organism in which they occur (1). The metabolic importance of peroxisomes in humans is demonstrated by the fact that the absence of the organelles leads to severe abnormalities, followed by an early death (*e.g.* Zellweger syndrome (2)). Consequently, many studies are now devoted to unravel the molecular mechanisms of peroxisome biogenesis and function. Yeasts are excellent model systems for such studies having the advantages that (i) the induction and protein composition of peroxisomes can readily be manipulated by varying growth conditions and (ii) in the absence of peroxisomes, yeasts are viable (3, 4). Hence, peroxisome-deficient mutants have been isolated from different yeast species (4), and the corresponding genes are being cloned and characterized.

In yeast, peroxisomes normally develop by growth and fission from pre-existing ones. Peroxisomal matrix proteins are nuclear-encoded, synthesized in the cytoplasm, and directed to the organelle by topogenic signals (PTSs).[1] Two PTSs have been identified and are located either at the extreme C terminus (PTS1) or the N terminus of the protein (PTS2) (4). Our knowledge on the sorting of peroxisomal membrane proteins is still limited, and consensus topogenic sequences have yet to be identified (5).

In our laboratory, we use the methylotrophic yeast *Hansenula polymorpha* as the model organism for studies on peroxisome biogenesis and function. We have isolated a collection of peroxisome-deficient (*per*) mutants of this organism that comprises 28 different complementation groups and that includes constitutive and conditional (temperature-sensitive) mutants. In previous reports, we described the cloning and characterization of three *H. polymorpha PER* genes, namely *PER1*, *PER3*, and *PER8* (6–8), the protein products of which are part of the protein import (Per1p and Per3p) (6, 7) or peroxisome proliferation machinery (Per8p) (8). Here, we describe the molecular cloning and characterization of the *PER9* gene and its protein product (Per9p). Per9p shares sequence similarity with *Saccharomyces cerevisiae* Pas3p (9) and plays a key role in peroxisome biogenesis and maintenance.

EXPERIMENTAL PROCEDURES

Organisms and Growth Conditions—Escherichia coli strain DH5α was cultivated as described (10). *H. polymorpha* was grown in batch cultures at 37 °C on (*a*) selective minimal medium containing 0.67% yeast nitrogen base without amino acids supplemented with 1% glucose or 0.5% methanol (YNM); (*b*) medium containing 1% yeast extract, 2% peptone, and 1% glucose; or (*c*) mineral medium (11) supplemented with 0.5% carbon source and 0.25% nitrogen source. Carbon sources used were glucose, glycerol, ethanol, and methanol; as nitrogen sources, methylamine, D-alanine, and ammonium sulfate were added. In addition, cells were grown in continuous cultures at 37 °C in mineral medium (11) on a mixture of 0.25% glucose and 0.25% methanol. When

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[1] The abbreviations used are: PTS, peroxisomal topogenic signals; kb, kilobase; P_{AOX}, alcohol oxidase promoter; P_{AMO}, amine oxidase promoter; P_{PER9}, *PER9* promoter; AO, alcohol oxidase; CAT, catalase; ER, endoplasmic reticulum.

needed, amino acids and uracil were added to a final concentration of 40 μg ml^{-1}. For growth on agar plates, all media were supplemented with 1.5% granulated agar. The following *H. polymorpha* strains were used in this study: (i) *per9-1* (*leu1.1 per9*) (12), (ii) NCYC495 (*leu1.1 ura3*), (iii) NCYC495 (*leu1.1*) (13), (iv) NCYC495 *cat::LEU2* (*ura3* Δ*cat*),[2] (v) *per9::LEU2* (*ura3* Δ*per9*), and (vi) *per9::URA3* (*leu1.1* Δ*per9*) (this study).

Isolation and Characterization of the PER9 Gene—Genetic manipulations of *H. polymorpha* were performed as described previously (12–15). *H. polymorpha* was transformed as detailed by Faber *et al.* (16). Standard recombinant DNA techniques were carried out essentially as described (10).

To clone the *PER9* gene, mutant *per9-1* was transformed with an *H. polymorpha* genomic library constructed in vector pYT3 (8). Leucine prototrophic transformants were screened on YNM plates for the ability to grow on methanol. The complementing plasmids of positive clones were rescued and transformed to *E. coli* DH5α (15). To facilitate sequencing, restriction analysis, and construction of subclones, a 2.7-kb complementing DNA fragment was subcloned as a *Sal*I fragment into *Sal*I-digested phagemid pBluescript II KS$^+$ (Stratagene, La Jolla, CA) in both orientations. Sequencing of both strands was carried out on an Applied Biosystems 373A automatic sequencer using the *Taq* dye deoxy terminator cycle sequencing kit supplied by the manufacturer. For DNA and amino acid sequence analysis, the PC-GENE® program (Release 6.70, IntelliGenetics, Mountain View, CA) was used. The TBLASTN algorithm (17) was used to search the GenBank™ Data Bank (Release 91.0, October 15, 1995) for DNA and protein sequences showing similarity to the *PER9* gene and its protein product.

PER9 Disruption—For disruption of the *PER9* gene, either the *Candida albicans LEU2* gene (obtained from Dr. E. Berardi, University of Ancona, Ancona, Italy) or the *H. polymorpha URA3* gene (18) was used. The *LEU2* gene was isolated as an *Eco*RI (blunt-ended)-*Bam*HI fragment and ligated between the *Bgl*II and *Stu*I sites of the 2.7-kb *PER9* fragment in pBluescript II KS$^+$, deleting half of the *PER9* gene. The *URA3* gene was isolated as a *Bam*HI (blunt-ended) fragment and ligated between the same *Bgl*II (blunt-ended) and *Stu*I sites. Disruption fragments (see Fig. 2*B*) were isolated by digestion with *Tth*I (*per9::LEU2*) or by partial digestion with *Sac*I (*per9::URA3*) and used to transform *H. polymorpha* NCYC495 (*leu1.1 ura3*). Methanol utilization-defective strains (Mut$^-$) were selected, and correct integration of *LEU2* and *URA3* was determined by Southern blot analysis using the ECL direct nucleic acid labeling and detection system (Amersham Corp.). Selected *per9* disruption strains (Δ*per9*) were also tested for complementation by the *per9-1* complementing fragment. To identify possible peroxisomal membrane remnants in Δ*per9* cells, a *per9::URA3* disruption strain was transformed with pET4 (8), which contains the *H. polymorpha PER8* gene under the control of the alcohol oxidase promoter (P_{AOX}).

Construction of PER9-Catalase Fusions—Fusions were constructed between *PER9* and an *H. polymorpha* catalase gene from which the PTS1 function was destroyed by changing the last triplet coding for isoleucine to one coding for lysine (*CAT(−PTS1)*) (19). A *Hin*dIII site (5′-primer, 5′-GGAAGCTTATGTCAAACCCCCCTGTTT-3′) was introduced by polymerase chain reaction directly 5′ of the ATG start codon of the mutant *CAT* gene present on plasmid pHCAT-K (a gift from Dr. R. Roggenkamp, Heinrich Heine University, Düsseldorf, Germany). The *CAT(−PTS1)* gene was cloned in frame downstream fragments encoding selected portions of Per9p using (i) the *Hin*dIII (N_{16}Per9p-CAT(−PTS1); *Hin*dIII site of *CAT(−PTS1)*), (ii) *Nhe*I (blunt-ended) (N_{37}Per9p-CAT(−PTS1); *Hin*dIII site of *CAT* blunt-ended), and (iii) *Hin*dII (N_{115}Per9p-CAT(−PTS1); *Hin*dIII terminus of *CAT* made blunt-ended by mung bean exonuclease sites in the *PER9* gene. Plasmid pHIPX2 (20) into which the *H. polymorpha URA3* gene had been inserted was used to place the chimeric *PER9-CAT(−PTS1)* genes behind P_{AOX}. The resulting plasmids were used to transform an *H. polymorpha* catalase disruption mutant (Δ*cat*).

Deletion of the Membrane-spanning Region of Per9p—Deletion of DNA sequences encoding the predicted membrane-spanning region of Per9p (amino acids 16–36) was carried out in several steps. First, the *Hin*dIII site in the 2.7-kb *per9* complementing fragment (in pBluescript II KS$^+$) was cut, followed by treatment with Klenow polymerase and religation, thereby generating a new *Nhe*I site. Next, the resulting plasmid was *Nhe*I-digested and religated. This resulted in the deletion of a *Nhe*I fragment encoding 21 amino acids (amino acids 16–36). A fragment containing the *PER9* promoter and the mutant *PER9* gene was subcloned into pHIPX3 after digestion of both plasmids with *Bam*HI and *Sal*I. The pHIPX3 shuttle vector was constructed by cloning a 5.7-kb *Bam*HI-*Nru*I (blunt-ended) fragment from pHIPX1 (20) into *Bam*HI- and *Stu*I-digested pOK12 (21). The final plasmid carrying the mutant *PER9* gene was introduced in the *per9::URA3* disruption mutant and analyzed for its ability to restore peroxisome formation.

Construction of PER9 Conditional Mutants—To study the effect of overexpression of *PER9*, the plasmid pHIPX4-PER9 (22), containing *PER9* behind P_{AOX}, was transformed into *H. polymorpha leu 1.1*. To study the reappearance of peroxisomes in relation to the reintroduction of Per9p, the *PER9* gene was placed under the control of the substrate-inducible *H. polymorpha* amine oxidase promoter (P_{AMO}). P_{AMO} is fully repressed by ammonium ions, but is induced by primary amines (23, 24). Plasmid pHIPX5-PER9 (22), containing *PER9* under the control of P_{AMO}, was linearized with *Sau*I and transformed to the *per9::URA3* disruption mutant. Correct integration of the plasmid into the genome at the P_{AMO} locus was confirmed by Southern blot analysis.

Generation of Antisera—For synthesis and purification of Per9p, the protein fusion and purification system supplied by New England Biolabs Inc. (Beverly, MA) was used. An *Aha*II-*Sal*I *PER9* fragment (in pBluescript II KS$^+$) was subcloned in frame behind the *mal*E gene in pMAL-C2 digested with *Eco*RI and treated with mung bean exonuclease and then cleaved with *Sal*I. Expression of the *mal*E-*PER9* chimeric gene under the control of the *tac* promoter was induced by addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside to exponentially growing cultures. The hybrid protein was purified according to the manufacturer (New England Biolabs Inc.) and used for immunization of a rabbit.

Expression Levels of the PER9 Gene—The *PER9* expression levels were studied using β-lactamase as reporter protein in *H. polymorpha* wild-type cells. For this purpose, the *E. coli* β-lactamase gene was cloned in frame with part of the *PER9* gene, encoding the first 7 amino acids (using the *Bgl*II site), expressed under the control of P_{PER9} (for details, see Ref. 6).

Biochemical Methods—Crude extracts were prepared according to Ref. 25. Cell fractionation was performed as described previously (26), except that 1 mM phenylmethylsulfonyl fluoride and 2.5 μg/ml leupeptin were added to all solutions. Peroxisomal peak fractions were subjected to carbonate extraction (27) or high salt treatment (incubation in 0.5 M sodium chloride in 50 mM potassium phosphate, pH 7.5, for 30 min at 4 °C, followed by centrifugation for 30 min at 100,000 × *g* and 4 °C). Enzyme activities of alcohol oxidase (28), catalase (29), and β-lactamase (6) were assayed by established procedures. Protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis was carried out as described (30). Gels were stained with Coomassie Brilliant Blue R-250 or subjected to Western blotting (31). The blots were decorated using the Protoblot immunoblotting system (Promega) and specific polyclonal antibodies against selected *H. polymorpha* peroxisomal proteins.

Electron Microscopy—Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as detailed before (6). Immunolabeling was performed on ultrathin sections of Unicryl-embedded cells using specific antibodies against various peroxisomal proteins and gold-conjugated goat anti-rabbit antibodies (6).

RESULTS

Cloning of PER9 by Functional Complementation—The original nitrosoguanidine-induced *per9-1* mutant of *H. polymorpha* was unable to grow on methanol (Mut$^-$ phenotype) and lacked peroxisomes (Fig. 1*A*). The *PER9* gene was cloned after transformation of the *per9-1* mutant with an *H. polymorpha* genomic DNA library using restoration of the Mut$^+$ phenotype as selection criterion.

From 10^5 colonies, three Mut$^+$ transformants were selected, each of which carried the library vector pYT3 with a 7.5-kb insert. By subcloning, the complementing activity was found to reside within a 2.7-kb *Sal*I fragment. Sequence analysis of the 2.7-kb complementing fragment revealed two open reading frames of 1374 and 621 base pairs (Fig. 2*A*). Further subcloning analysis demonstrated that the 1374-base pair open reading frame represented the complementing gene. This 1374-base pair open reading frame, hereafter referred to as the *PER9* gene, encoded a putative protein (Per9p) of 457 amino acids with a calculated mass of 52 kDa. Hydropathy analysis (32) predicted one membrane-spanning region (amino acids 16–36)

[2] M. Tuijl, unpublished results.

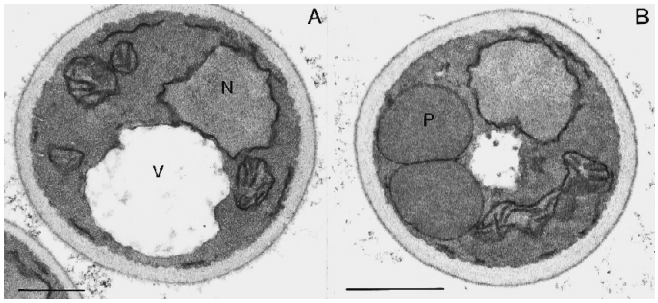

FIG. 1. **Ultrathin sections of methanol-incubated cells of the original *per9-1* mutant and the wild-type strain of *H. polymorpha*.** The *per9-1* mutant (*A*) lacks peroxisomes, which are evident in the wild-type cell (*B*) ($KMnO_4$). Electron micrographs are taken from aldehyde-fixed cells, unless otherwise indicated. *Bar* = 0.5 μm.

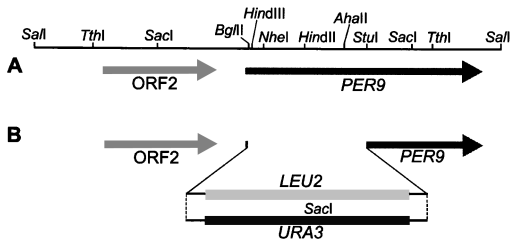

FIG. 2. **Schematic representations of the 2.7-kb *per9-1* complementing fragment containing open reading frame 2 (*ORF2*) and the *PER9* gene (*A*) and the disruption of *PER9* using *C. albicans LEU2* or *H. polymorpha URA3* (*B*).** The *Sal*I site to the left originates from vector pYT3. The nucleotide sequence has been deposited in the GenBank℠ Data Bank under accession number U37763.

and one membrane-associated region (amino acids 159–179). A data base search revealed strong similarity to the integral peroxisomal membrane protein Pas3p of *S. cerevisiae* (Fig. 3) (9). *H. polymorpha* Per9p and *S. cerevisiae* Pas3p display a similar hydropathy pattern, and both have one predicted membrane-spanning region and one membrane-associated region.

Construction and Characterization of a PER9 Disruption Mutant—To study the effect of the absence of Per9p on peroxisome biogenesis, an *H. polymorpha per9* disruption strain was constructed in which 691 base pairs of *PER9* (amino acids 7–236) were deleted and replaced with a DNA fragment encoding the *C. albicans LEU2* gene. This strain, Δ*per9*, showed absolute co-segregation of the Mut^- and Leu^+ phenotypes during random spore analysis of diploids obtained after mating the strain with auxotrophic *H. polymorpha* wild-type strains. Diploids obtained from crossing of the Δ*per9* strain with the *per9-1* mutant were Mut^-. Upon sporulation, the resulting haploids all were Mut^- as well. Together, these genetic results demonstrated that *per9-1* and Δ*per9* were closely linked and most likely alleles of the same gene. Thus, the cloned DNA fragment encodes the gene that is defective in *per9-1* and not a suppressor gene.

The Δ*per9* strain grew at a wild-type rate on rich medium (*e.g.* glucose/ammonium sulfate) and also on selected carbon (*e.g.* ethanol) and nitrogen (*e.g.* D-alanine and methyl- and ethylamine) sources known to require the activity of peroxisomal enzymes (33). However, growth on methanol as sole source of carbon and energy was totally defective (data not shown). Electron microscopic analysis of thin sections of Δ*per9* cells demonstrated that under all growth conditions employed, intact peroxisomes were invariably absent (data not shown). The subcellular morphology of Δ*per9* was studied in cells grown in a continuous culture on a glucose/methanol mixture. Enzyme activity measurements (data not shown) and Western blot analysis (Fig. 4) revealed that each peroxisomal protein examined (alcohol oxidase (AO), catalase (CAT), dihydroxyacetone

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HpPer9p    MFQYCRDLVSRHKKKLLFGTGVIAVSYAVSS----FVSNKLAELNERLKEENF  49
ScPas3p    MAPNQRSRSLLQRHRGKVLISLTGIAALFTTGSVVVFFVKRWLYKQQLRITEQHF  55

HpPer9p    AKEQIKRRFKQTQNDCYMTFLSLLPVLTEPIYE-ALKVEEITRELQNKRFERQKA 103
ScPas3p    IKEQIKRRFEQTQEDSLYTIYELLPVWRMVLNENDLNLDSIVTQLKDQKNQLTRA 110

HpPer9p    KNAPVGNSTDPALSTVLSDDFSVHNEKENPMHTGVQQTKSKTQLWNELRNQSITR 158
ScPas3p    KSSESRESS----------------------------PLKSKAELWNELELKSLIK 138

HpPer9p    FLTLLYCESLLIVFLHLQLNILSRRSYLETAIKLASKTKGIKLENESNVDL---- 209
ScPas3p    LVTVTYTVSSLILLTRLQLNILTRNEYLDSAIKLTMQQENCNKLQNRFYNWVTSW 193

HpPer9p    --DPANLFLENDEELAMGSSRQDEN--LAEQAFLSYSWWLLNKGWIDIKNQVESS 260
ScPas3p    WSDPEDKADDAMVMAAKKSKKEGQEVYINEQAFLSLSWWILNKGWLSYNEIITNQ 248

HpPer9p    VEDIFGDINPRQELSIEEFATLINKTQQIIDKQIYAEKESEPLTAPGHSSSTVIT 315
ScPas3p    IEIEFDGIHPRDTLTLEEFSSRLTNIFRNTNSQIFQQNNNN----------LTS 292

HpPer9p    SLLPPANM-ELFLLQQTNDMEFLTNFNNNIQNSENVSKLLNELKGYLMNDQVSAI 369
ScPas3p    ILLPKDSSGQEFLLSQTLDADALTSFHSNTLV---FNQLVNELTQCIESTATSIV 344

HpPer9p    ISLLVTVGISKVLDNIVVN-LMNKQKNPDQEEQVPTEIPKVKLASLLASITKQSN 423
ScPas3p    LESLINESFHFIMNKVGIKTIAKKKPGQEDQQQYQMAVFAMSMKDCCQEMLQTTA 399

HpPer9p    QLTNNSLDNEVLYTLNNLQELDDLSASVYSNFDA                      457
ScPas3p    GSSHSGSVNEYLATLDSVQPLDDLSASVYSNFGVSSSFSFKP              441
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FIG. 3. **Amino acid sequence alignment of *H. polymorpha* Per9p (*HpPer9p*) and *S. cerevisiae* Pas3p (*ScPas3p*).** The putative membrane-spanning region and membrane-associated region in both proteins are *underlined*. Identical residues are indicated by *asterisks*, and similar ones by *dots*. Gaps were introduced to maximize the similarity.

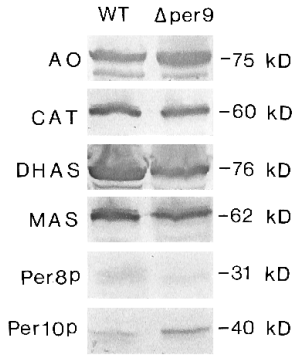

FIG. 4. **Protein levels of peroxisomal matrix and membrane proteins in *H. polymorpha* Δ*per9* and wild-type cells.** Western blots are prepared of crude extracts from methanol-grown wild-type (*WT*) or Δ*per9* cells and decorated with antibodies against *H. polymorpha* AO, CAT, dihydroxyacetone synthase (*DHAS*), malate synthase (*MAS*), Per8p, and Per10p. 30 (AO, CAT, dihydroxyacetone synthase, and malate synthase), 40 (Per10p), and 60 (Per8p) μg of protein were loaded on each lane.

synthase, malate synthase, Per8p (8), and Per10p[3]) was present at normal wild-type levels. Ultrastructural analysis showed that peroxisomes were absent. As we have observed in other *H. polymorpha per* mutants (34), the Δ*per9* cells contained a large cytoplasmic crystalloid (Fig. 5*A*). Immunocytochemistry indicated that the other matrix proteins were also not in peroxisomes, but were located in the cytoplasm (shown for AO and CAT in Fig. 5, *B* and *C*, respectively; the wild-type CAT control is shown in Fig. 5*F*). Membranous remnants of peroxisomes (vesicles, ghosts) were not observed. In addition, overproduction of the *H. polymorpha* peroxisomal membrane protein Per8p (8) also failed to resolve such structures. Instead, Per8p was found in small aggregates that were often associated with a mitochondrial profile (Fig. 5*E*). Since peroxisomal vesicles are readily detected in other *per* disruption strains overexpressing

[3] M. Komori and M. Veenhuis, unpublished results.

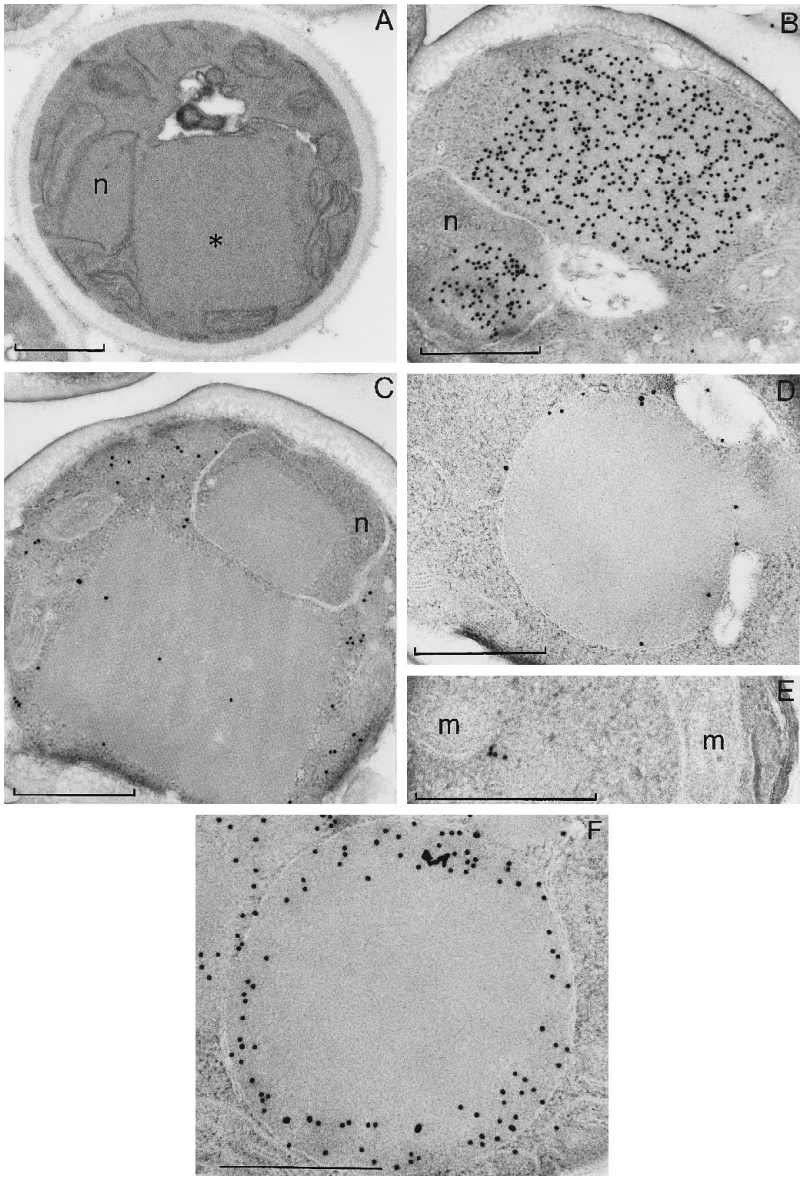

FIG. 5. *A*, shown is the overall morphology of the *per9::LEU2* disruption mutant. Cells were incubated for 36 h in batch cultures containing 0.5% methanol. Peroxisomes were absent; instead, a large cytosolic crystalloid was present (*). *B*, these crystalloids were labeled with anti-AO antibodies, including the crystalloids in the nucleus. *C*, CAT was predominantly localized in the cytoplasm (anti-CAT antibodies). *D*, shown is the immunocytochemical localization of Per9p on the peroxisomal membrane (anti-Per9p). *E*, shown is a characteristic picture of the labeling obtained after incubation of ultrathin sections of Δ*per9* cells, overexpressing the *H. polymorpha PER8* gene, with specific antibodies against Per8p. Peroxisomal ghosts were never seen in such cells; instead, low but specific Per8p labeling was observed, generally associated with a mitochondrial profile. *F*, shown is the typical labeling pattern of catalase protein in peroxisomes of *H. polymorpha* wild-type cells. *m*, mitochondrion; *n*, nucleus. *Bar* = 0.5 μm.

PER8 (*e.g.* Δ*per1*) (6, 35), we concluded that membranous remnants were either absent in Δ*per9* cells or present at a level below the limit of detection of the ultrastructural methods.

Subcellular Location of Per9p—The subcellular location of Per9p was studied by both conventional cell fractionation and ultrastructural techniques. Antibodies were raised against Per9p synthesized in *E. coli*. On Western blots decorated with anti-Per9p antiserum, a dominant protein band of ~59 kDa was found in crude extracts of *H. polymorpha* wild-type and *PER9*-overexpressing cells (Fig. 6*A*). Western blot analysis of organellar peak fractions, obtained after sucrose density centrifugation of organellar pellets prepared from homogenates of methanol-grown wild-type *H. polymorpha*, showed that Per9p was pelletable and cosedimented with the peroxisomal peak fraction, characterized by the presence of Per8p (Fig. 6*B*). After high salt treatment of the peroxisome-enriched fractions and subsequent centrifugation, Per9p was present in the pellet. Similarly, after carbonate treatment, the major portion of Per9p was sedimentable (Fig. 6*C*). CAT, used as control, behaved like a soluble protein, while Per8p was pelletable (Fig. 6*D*). These data indicate that Per9p is an integral peroxisomal membrane protein. This location was confirmed immunocytochemically using antibodies against Per9p (Fig. 5*D*).

To gain information on the topogenic signals of Per9p, we expressed selected chimeric genes composed of *PER9* sequences encoding different N-terminal parts of Per9p fused to a *CAT* gene encoding CAT lacking a functional PTS1 (CAT(−PTS1)). In previous studies, we showed that this PTS1-mutated *CAT* gene product is not sorted to peroxisomes of *H. polymorpha*, but instead accumulates in the cytosol (36). The fusion products were synthesized in an *H. polymorpha CAT* disruption strain (Δ*cat*).[2] Immunocytochemical experiments performed on a strain producing the first 16 amino acids (N_{16}) of Per9p fused to CAT(−PTS1) revealed that the N_{16}Per9p-CAT(−PTS1) hybrid protein was located at membranous layers, most probably derived from the ER, since also the nuclear envelope often showed labeling (Fig. 7, *A* and *B*). In addition, low labeling intensities were found on the peroxisomal membrane (Fig. 7*A*). When larger parts of Per9p (N_{37} or N_{115}) were used for fusion, labeling was predominantly on the peroxisomal membrane (data not shown), indicating that additional information is required for Per9p to insert in the peroxisomal membrane. Infrequently, small vesicles were seen associated with these membranes, which were also labeled (Fig. 7*B*). CAT protein was not, or to a very low extent as judged by the labeling patterns, in the peroxisomal matrix; it must be

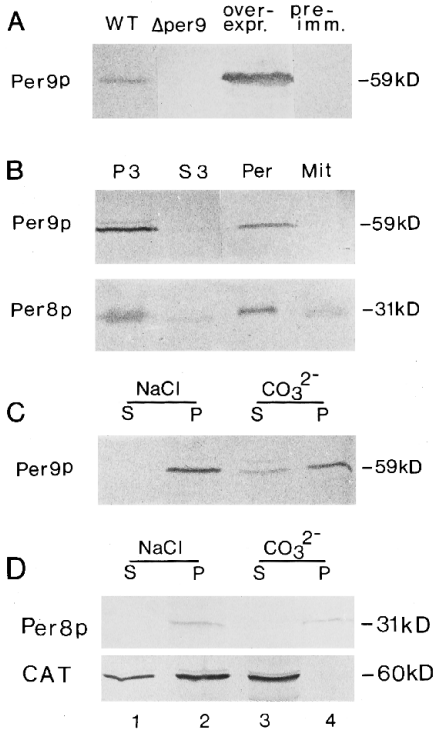

FIG. 6. *A*, shown is the specificity of the anti-Per9p antiserum. Western blots were prepared from crude extracts of *H. polymorpha* wild-type cells (*WT*; *lanes 1* and *4*), Δ*per9* cells (*lane 2*), and cells overexpressing the *PER9* gene (*lane 3*). No signal was observed when the preimmune serum (*lane 4*) was used. Cells were grown/incubated in batch cultures on 0.5% methanol. Equal amounts of protein were loaded per lane. *B*, shown is the localization of Per9p. The 30,000 × *g* supernatant (*S3*; *lane 2*) and organellar pellet (*P3*; *lane 1*), obtained after differential centrifugation of homogenates prepared from methanol-grown wild-type cells of *H. polymorpha*, as well as the peroxisomal (*Per*; *lane 3*) and mitochondrial (*Mit*; *lane 4*) fractions, obtained after subsequent sucrose density centrifugation of the P3 organellar pellet, were subjected to Western blotting. The data show that Per9p is a peroxisomal protein. As a control, the *H. polymorpha* integral peroxisomal membrane protein Per8p was used. *C*, shown are Western blots demonstrating the distribution of Per9p over the soluble (*S*; *lanes 1* and *3*) and pelletable (*P*; *lanes 2* and *4*) fractions after high salt treatment (*NaCl*; *lanes 1* and *2*) and sodium carbonate extraction (CO_3^{2-}; *lanes 3* and *4*) of the peroxisome-enriched peak fractions. *D*, as controls for the location of Per9p (see *C*), the distribution of the matrix protein CAT and Per8p (an integral component of the peroxisomal membrane of *H. polymorpha*) was studied. Per8p was pelletable after both high salt treatment (*NaCl*; *lanes 1* and *2*) and sodium carbonate extraction (CO_3^{2-}; *lanes 3* and *4*) of the peroxisomal peak fractions; instead, catalase was partly solubilized after NaCl treatment (*lanes 1* and *2*), but completely soluble after carbonate treatment of these fractions (*lanes 3* and *4*). These data confirm that Per9p is an integral membrane protein of peroxisomes of *H. polymorpha*.

emphasized that the immunocytochemical methods allow us to discriminate between an intraperoxisomal and membrane-bound location in batch-cultured cells (37). From these results, we conclude that the first 16 amino acids of *H. polymorpha* Per9p contain topogenic information that is able to direct a reporter protein (CAT(−PTS1)) to the ER.

PER9 Is Induced under Peroxisome-inducing Conditions—To obtain insight in the regulation of *PER9* expression, the activity of P_{PER9} was studied in detail using P_{PER9}-driven synthesis of bacterial β-lactamase. This method has been successfully used for analysis of the regulation of low abundant peroxisomal proteins (6). The results, summarized in Fig. 8, indicate that P_{PER9} is active under all growth conditions tested. In glucose- and ethanol-grown cells, the activity of P_{PER9} was low. Enhanced β-lactamase activities were observed when the cells were grown on nitrogen sources that require peroxisomal enzymes (methylamine or D-alanine) (33). The highest activities were observed in cells grown on methanol. As a control for our assay system, we used an identical plasmid containing the β-lactamase gene expressed under the control of P_{AOX} (6). No β-lactamase activity was detected in cells grown on glucose or ethanol, conditions known to fully repress P_{AOX} (33). As shown before (6), these results indicate that carbon catabolite repression of P_{AOX} operates effectively on multicopy autonomously replicating plasmids. As expected, high activities were observed in methanol-grown cells. In methanol-grown cells, P_{AOX}-driven β-lactamase activity was ~10-fold higher than the activity observed under the control of P_{PER9}. This indicates that *PER9* is expressed at moderate levels in methanol-grown cells of *H. polymorpha*.

Functional Properties of Per9p—To gain insight in Per9p function, a conditional mutant was constructed in which the level of *PER9* expression could be manipulated by varying the growth conditions. For this purpose, the *PER9* gene was placed under the control of the inducible amine oxidase promoter. In the absence of amine substrate during growth of cells on ammonium sulfate, P_{AMO} is repressed. The P_{AMO}-*PER9*-containing plasmid was integrated into the genome of a Δ*per9* strain. As expected, these cells did not contain recognizable peroxisomes during exponential growth on glucose/ammonium sulfate, but contained normal peroxisomes on methanol/methylamine-containing medium (data not shown). This result is consistent with our results on Δ*per9* showing that Per9p is essential for peroxisome biogenesis.

We examined the kinetics of the biogenesis of peroxisomes by electron microscopy in P_{AMO}-*PER9*-containing Δ*per9* cells after a shift from ammonium sulfate- to methylamine-containing medium. These experiments indicated that new small peroxisomes were first detected ~30 min after the shift. Typically, only one small organelle per cell initially developed, which was characterized by the presence of AO and Per9p (Fig. 9, *A* and *B*). These organelles subsequently increased in size and multiplied during further cultivation, as described before for a shift of wild-type cells from glucose to methanol (38). The mechanisms of peroxisome reintroduction in these cells are currently being studied in depth and will be detailed in a separate paper.

Subsequently, cells grown on methanol/methylamine were shifted to methanol/ammonium sulfate-containing medium, thus repressing Per9p synthesis, and the effect on peroxisome morphology was followed by electron microscopy. In the initial hours after the shift, morphological alterations of existing organelles were not detectable. After ~8 h of incubation, a partial disintegration of the peroxisomal membranes was observed (Fig. 9*C*). This result was highly reproducible with regard to both the time interval (±8 h) and the deterioration effect on the membrane. At this stage, soluble AO protein was also first observed in the cytosol (Fig. 9*D*). At later stages, AO crystalloids that lacked a surrounding membrane appeared in the cells; a number of these crystalloids were subsequently degraded in the vacuole (data not shown). These results indicate that Per9p plays a role in maintenance of the peroxisomal membrane *in vivo*.

Per9p may also play a role in matrix protein assembly. This aspect was further investigated in Δ*per9* cells transformed with an expression plasmid carrying a truncated *PER9* lacking the region coding for the predicted membrane-spanning region (amino acids 16–36). These transformants could not grow on methanol and lacked peroxisomes, suggesting that the peroxisomal location of Per9p is essential for its functioning. Surprisingly, AO was not assembled and active in these cells, as it is in Δ*per9* cells, but instead, AO was present in cytoplasmic aggregates in which the truncated Per9p protein was also located

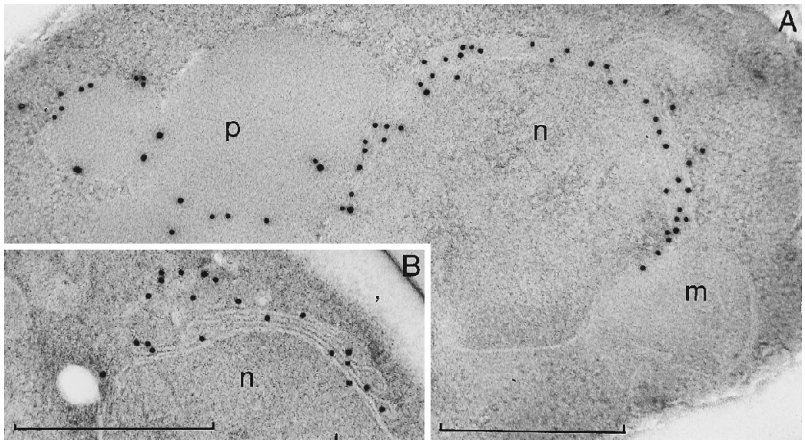

FIG. 7. **Shown in *A* is the typical labeling patterns after incubation of ultrathin sections of *H. polymorpha* Δ*cat* cells, synthesizing N_{16}Per9p-CAT(−PTS1), with specific anti-CAT antibodies.** Labeling is predominantly located on layers of membranes, associated with the nucleus, and on the peroxisomal membrane. In the *inset* (*B*), labeled small vesicles are seen. *m*, mitochondrion; *n*, nucleus; *p*, peroxisome. *Bar* = 0.5 μm.

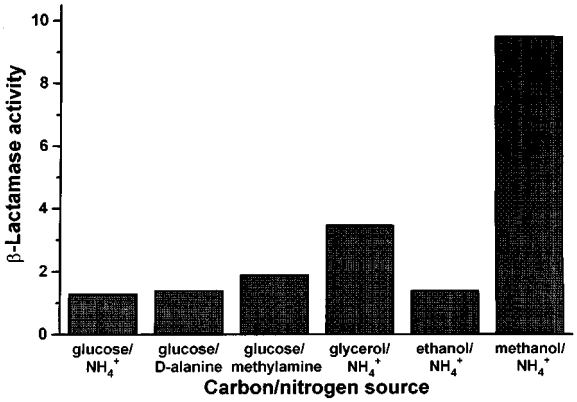

FIG. 8. **Activity of the *PER9* promoter determined by P_{PER9}-driven β-lactamase synthesis under selected growth conditions.** β-Lactamase enzyme activities are expressed as units/milligram of protein.

(Fig. 9, *E* and *F*).

Finally, we studied the effect of *PER9* overexpression on peroxisome biogenesis. An *H. polymorpha* strain was constructed that expresses *PER9* under the control of the strong AO promoter. Electron microscopic analysis of such cells grown on methanol revealed that they contained higher than normal numbers of peroxisomes, often accompanied by vesicular structures (Fig. 9*G*).

DISCUSSION

PER9 Is an Integral Membrane Protein Essential for Peroxisome Biogenesis—We have identified and characterized the *PER9* gene encoding an integral peroxisomal membrane protein of the methylotrophic yeast *H. polymorpha*. Per9p shares 30% sequence identity with the *S. cerevisiae* Pas3p (9). Synthesis of Pas3p in an *H. polymorpha per9* disruption mutant (Δ*per9*) restores the Mut^+ phenotype of Δ*per9* cells, which also contain intact peroxisomes, again indicating that Pas3p and Per9p are functional homologues (22). Höhfeld *et al.* (9) suggested that the conserved N-terminal hydrophobic region of Pas3p is anchored in the peroxisomal membrane, while the C-terminal portion of the protein is exposed to the cytosol. On the basis of the interchangeable functions, we assume a similar topology for Per9p in the peroxisomal membrane of *H. polymorpha*.

Disruption of the *PER9* gene had a drastic effect on the overall morphology of derepressed *H. polymorpha* cells in that (i) peroxisomes are absent and (ii) peroxisomal membrane remnants are also not detected. Peroxisomal matrix proteins like AO, CAT, dihydroxyacetone synthase, and malate synthase are normally synthesized and active in the cytosol as in other *per* disruption strains; peroxisomal membrane proteins (Per8p (8) and Per10p[3]) were also present at approximately normal levels in Δ*per9* cells. However, vesicular structures ("ghosts") (39) were never observed. The method used (overproduction of the *H. polymorpha* peroxisomal membrane protein Per8p as marker protein) enabled such vesicles in other *H. polymorpha* Δ*per* strains to be readily discerned (35). By a comparable method, Purdue and Lazarow (40) demonstrated the presence of peroxisomal membrane vesicles in *S. cerevisiae peb* mutants. The apparent absence of peroxisomal vesicles in Δ*per9* cells makes this mutant attractive for molecular studies on the reintroduction of peroxisomes as occurs in transformants that synthesize Per9p under the control of a substrate-inducible promoter. This reintroduction is in line with earlier observations that peroxisomes in *H. polymorpha* do not necessarily derive from pre-existing peroxisomes (41). The mechanisms of the reintroduction of peroxisomes in *per* disruption strains, including Δ*per9*, are currently being studied in our laboratory.

What Is the Function of PER9?—Our results indicate that Per9p is a key component of the peroxisome assembly machinery and in fact may have multiple functions. One of these is that the protein plays a role in maintenance of the peroxisomal membrane, as was evident after the repression of *PER9* expression in a constructed conditional mutant. Probably, the integrity of peroxisomal membranes in *H. polymorpha* requires a continuous addition of newly synthesized Per9p.

Moreover, Per9p seems to be involved in peroxisome proliferation. The multiplication of organelles, observed under conditions of *PER9* overexpression, could simply reflect a Per9p-mediated enhanced synthesis of peroxisomal membranes. On the other hand, it is tempting to speculate that the mechanisms controlling peroxisome proliferation and membrane biogenesis are functionally related in that the proteins involved in these processes are associated (or only can function) in one complex. Such functional interactions were already predicted from a classical genetic study in which various *H. polymorpha* genes, including *PER9*, were shown to be functionally linked (12).

H. polymorpha Per3p may also play a role in this putative protein complex. Recently, Per3p was identified as the *H. polymorpha* PTS1 receptor (7), showing both functional and structural similarities to the *Pichia pastoris* PTS1 receptor, Pas8p (42, 43). We postulated a model in which Per3p acts as the cytosolic receptor of newly formed PTS1 proteins that shuttles these polypeptides from the cytoplasm into the organellar matrix. One of the missing links in this model is that it does not explain how the Per3p-PTS1 protein complex reaches the peroxisomal membrane. Per3p lacks any known PTS and does not enter the peroxisome after overexpression of *PER3* in *H. polymorpha* wild-type cells (7). Hence, we proposed that Per3p undergoes a conformational change due to or after binding to

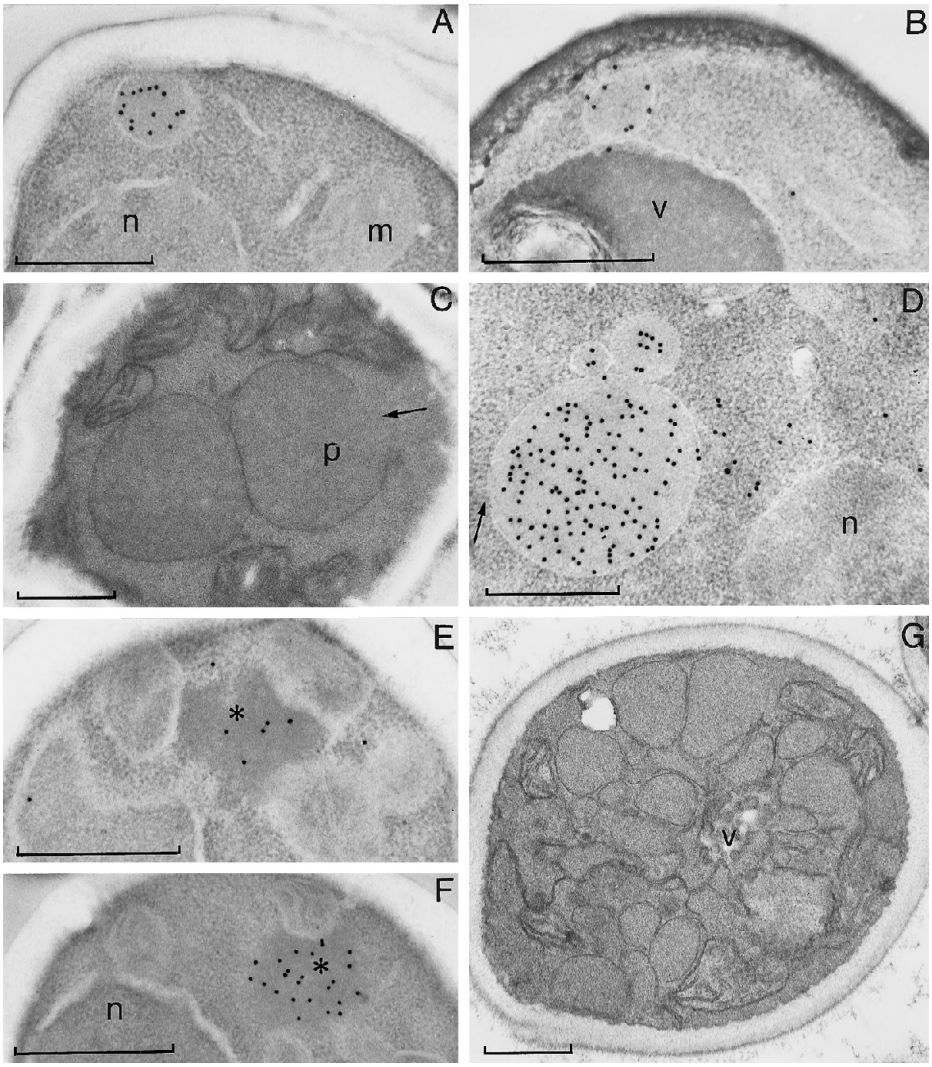

FIG. 9. *A* and *B* show details of cells of *per9::URA3* with genomically integrated P_{AMO}-*PER9* 4 h after the shift of cells from glucose/ammonium sulfate- to methanol/methylamine-containing medium. Small peroxisomes were present in the cells, characterized by the presence of AO (*A*; anti-AO) and Per9p (*B*; anti-Per9p). After incubation for 12 h in methanol/methylamine, the cells were transferred back to methanol/ammonium sulfate, thus repressing Per9p synthesis. 8 h after the shift, the first manifestation of disintegration of the peroxisomal membranes (*arrows*) was observed (*C* and *D*), associated with the appearance of AO protein in the cytoplasm (*D*; anti-AO antibodies). *E* and *F* show the presence of AO protein in cytosolic aggregates (*), present in Δ*per9* cells synthesizing truncated Per9p from which the membrane-spanning region is deleted. In these aggregates, besides AO (*F*; anti-AO antibodies), also truncated Per9p was present (*E*; anti-Per9p antibodies). *G* shows the presence of increased numbers of peroxisomes of various sizes in transformed methanol-grown *H. polymorpha* cells expressing a plasmid containing *PER9* under the control of the strong AO promoter. *m*, mitochondrion; *n*, nucleus; *p*, peroxisome; *v*, vacuole. *Bar* = 0.5 μm.

the PTS1 protein. The modified Per3p is subsequently recognized by a second protein that mediates sorting to the peroxisome. We speculate that Per9p could represent this protein; the involvement of Per9p in this process is also suggested by the simultaneous aggregation of AO and truncated Per9p lacking its membrane-spanning region. This hypothesis is in line with genetic studies by Titorenko *et al.* (12), who predict a functional interaction between Per9p and Per3p; it also does not conflict with the proposed function of Pas3p of *S. cerevisiae* (9), which was suggested to act as a protein receptor.

Unexpectedly, the first 16 amino acids of *PER9* were already sufficient to sort CAT, lacking its functional PTS, to the ER and nuclear membrane. One possible but unlikely explanation for this result is the cryptic nuclear targeting signal present in the 16 N-terminal amino acids of Per9p. The low labeling of CAT on peroxisomes can then be explained as a result of "piggybacking" (44, 45) in that the N_{16}Per9p-CAT fusion protein associates with authentic Per9p, which is also synthesized in these cells. The alternative is that the ER is directly involved in peroxisome biogenesis and that protein import and membrane biosynthesis are coupled processes. This can be envisaged in the view that peroxisomes are compartments, filled with proteins, that can only incorporate additional protein when the internal volume is simultaneously increased by recruiting phospholipids from the ER. The view that the ER may be involved in the biogenesis of peroxisomes is further supported by the finding that brefeldin A prevents peroxisome formation, resulting in the accumulation of peroxisomal matrix proteins at the ER.[4] It is interesting to note in this context that Bodnar and Rachubinski (46) described a 50-kDa integral membrane protein of mammalian peroxisomes that was synthesized on membrane-bound polysomes. It is still unclear, however, whether Per9p has properties similar to this protein.

The concept of protein import coupled to membrane insertion, *e.g.* via vesicle formation (45), of course leaves many questions unsolved, but takes its attractiveness from the fact that it unites a number of yet unexplained observations in various organisms in one model. Among these are the observations of Bellion and Goodman (47) on the import of AO in *Candida boidinii*. They showed that import and subsequent octamerization of AO was prevented in the presence of ionophores and resulted in the formation of peroxisomal membrane-associated protein complexes consisting of AO, dihydroxyacetone synthase, and several other unknown proteins (47). It can be envisaged that these proteins are not imported because membrane vesicle formation and/or fusion is prevented due to the lowered ATP levels in the cell. This mode of import could also explain how mature complex proteins or even gold particles enter the peroxisome (44, 45, 48). Further studies on the putative role of the ER in peroxisome biogenesis are currently in progress.

[4] F. A. Salomons, I. J. van der Klei, and M. Veenhuis, manuscript in preparation.

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